

Research Article

Photoprotective Effect of the Plant *Collaea argentina* against Adverse Effects Induced by Photodynamic Therapy

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Photodynamic therapy (PDT) is a treatment modality for tumours and other accessible lesions based on the combination of light and a photosensitizer (PS) accumulated in the target tissue. The main disadvantage of PDT is PS retention after treatment during long time periods that conduces to cutaneous damage. It is believed that singlet oxygen is responsible for that skin photosensitization. The aim of this work was to evaluate the photoprotective activity of the methanolic extract of the Argentinian plant *Collaea argentina* against PDT under several treatments and employing different PSs. *C. argentina* exhibited photoprotective activity against aminolevulinic acid- (ALA-) PDT in the LM2 murine adenocarcinoma cell line. The photoprotection was dependant on the extract concentration and the incubation time, being detectable from 40 $\mu\text{g/mL}$ onwards and at least after 3 h exposure of the cells. *C. argentina* extract protects these mammalian tumor cells against PDT effects, and it interferes with the oxygen singlet production from PSs during PDT treatment. We propose that it will be a promising agent to protect cells against PDT-induced skin sensitivity.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality for tumors and other accessible lesions. It is based on the combination of light and a photosensitizer (PS), which is a light-sensitive drug selectively accumulated in the target tissue [1].

It is widely accepted that photosensitivity is primarily caused by the production of reactive oxygen species (ROS). The cytotoxic effect of these species, through its reaction with cellular targets such as proteins, lipids, and DNA, is the rationale for the use of PDT in the treatment of cancer. Most clinical work on PDT has been carried out employing Photofrin, Temoporfin, or Verteporfin, that is, porphyrin derivatives.

5-Aminolevulinic acid (ALA) has also been successfully used as a tool for the photodiagnosis [2] and PDT of neoplastic tissue [3]. While ALA itself is neither fluorescent nor a photosensitizer, it can induce the biochemical formation of protoporphyrin IX (PpIX). Two molecules of ALA are converted into protoporphobilinogen, reaction mediated by protoporphobilinogen synthase, and 5 other enzymes, 3 cytosolic and 2 mitochondrial, which lead to the formation of PpIX, which is

a very efficient photosensitizer. Photodynamic action of PpIX is mainly induced by the generation of singlet oxygen in a type II photodynamic reaction [4].

ALA and other PSs currently employed in clinical PDT, such as Verteporfin and Temoporfin, produce singlet oxygen during the light exposure and in addition all the PSs are retained in other sites other than the tumor, thus inducing skin photosensitivity [4–7].

Photodynamic activity of certain plant components was first described about one hundred years ago. Bleaching of laundry on a lawn may be the earliest practical application of photodynamic activity. Chlorophyll, the green plant pigment, plays the main role in the production of this photodynamic action and, as well as some of its derivatives, it is used as a photosensitizer in PDT [8]. In addition, the anthraquinone, *Hypericum perforatum* extract, has recently been developed as a novel and natural PS for use in PDT of cancer. An analysis of a number of chemotherapeutic agents and their sources reveals that over 60% of approved drugs are derived from natural compounds [9].

Argentina has an abundant and diverse flora ranging from subarctic to subtropical climates; however, the medicinal properties of these plants have not been fully exploited. In previous work, we have carried out a screening of Argentinian plants in the search of new photosensitizers (PSs) [10].

All the methanolic leaf extracts showed photoactivity due to their chlorophyll or their derivatives content [10]. However, the extract of *C. argentina* leaves did not induce photodamage. Since this extract contains chlorophyll and its derivatives, we hypothesised that this lack of photosensitization could be due to the presence of photoprotective compounds. The aim of this work was to evaluate the photoprotective activity of *C. argentina* leaf extract against PDT under several conditions using different PSs.

2. Materials and Methods

2.1. Chemicals. ALA and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma-Aldrich (Poole, UK). Verteporfin was obtained from Conifarma, Argentina. Temoporfin was obtained from Biolite Pharma (Ireland, UK). The rest of the chemicals employed were of analytical grade. Chlorin e6 was from Frontier Scientific, USA, and Toluidine blue was from Merck.

2.2. Cell Line and Cell Culture. The cell line LM2 was derived from a spontaneous murine mammary adenocarcinoma of BALB/c mice. It was obtained from Instituto Roffo, Buenos Aires, Argentina [11]. The cells were cultured in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 80 µg/mL gentamycin, and 5% fetal bovine serum and were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

2.3. Plant Material. *Collaea argentina* Griseb. (BAA 2664) was collected from the Botanical Garden Lucien Hauman of the Agronomy School, University of Buenos Aires, and was identified by Ing. Agr. Juan Manuel Valla. A voucher specimen is kept in the herbarium of the mentioned institution. Plant nomenclature is according to Zuloaga et al. [12].

2.4. Extraction Procedure. Fresh leaves of *C. argentina* (100 to 200 g) were washed with distilled water, dried, and homogenized in absolute methanol. Methanolic extracts of *C. argentina* were filtered and evaporated under reduced pressure using a rotary evaporator and lyophilized afterwards to remove any traces of solvent. The obtained yields were 3% to 5%, and the resulting powders were stored at -20°C.

2.5. PDT Treatment. The cells were exposed to the different PSs (1 mM ALA, 3 mM Verteporfin, or 3 mM Temoporfin) in medium without serum. After 3 h exposure, the cells were irradiated for different time periods employing a light source located below the plate, at a distance of 20 cm. Afterwards, the medium was replaced by medium containing serum and the plates were incubated for 19 hours at 37°C and the MTT assay was performed.

The light source employed was a bank of two fluorescent lamps (Osram L 36W/10). The spectrum of the light is

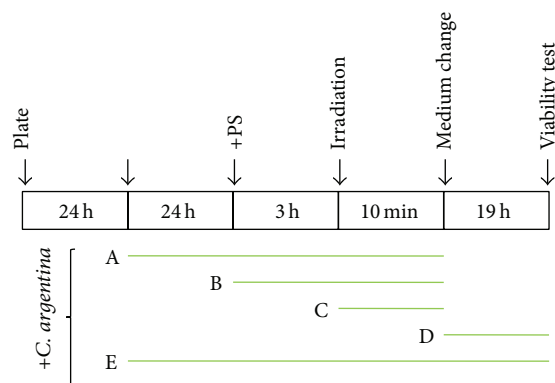


FIGURE 1: Scheme of the different treatments designed to evaluate the photoprotective effect of *C. argentina*.

between 400 and 700 nm with the highest radiant power at 600 nm. Fluence rate was measured with a Yellow Springs Kettering model 65 radiometer (Yellow Springs, OH, USA). We used fluences between 0 and 38 mJ/cm², corresponding to 0 to 10 min of irradiation.

2.6. Photoprotection Experiments. To test the possible photoprotective activity of *C. argentina*, the cells were plated at 5×10^4 cells/mL and 24 h afterwards, and they were exposed to different concentrations of *C. argentina*, before, during, and/or after PDT, according to Figure 1. *C. argentina* cytotoxicity (CI_{XX}) was defined as the concentration to kill XX% of cells.

2.7. Singlet Oxygen Production. To detect singlet oxygen (¹O₂), the shift of fluorescence of the fluorescent marker "Singlet Oxygen Sensor Green" (Invitrogen, USA) was used. The probe was added to the PSs solutions in PBS and the mixtures were irradiated with a 635 nm Lumina Laser system (Buenos Aires, Argentina) coupled to a fibre optic at 500 W during different times, ranging from 2 to 10 min. The fluorescent product was quantified at 504 and 525 nm excitation and emission wavelengths, respectively, in a Perkin Elmer LS55 fluorometer (UK). *C. argentina* + PS mixtures kept in dark were employed as controls.

2.8. MTT Viability Assay. Cytotoxicity and phototoxicity were documented by the MTT assay [13], a method based on the activity of mitochondrial dehydrogenases. Following appropriate treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well in a concentration of 0.5 mg/mL, and plates were incubated at 37°C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was read at 560 nm.

2.9. Statistical Treatment. The values in the figures and tables are expressed as means \pm standard deviations of the means. A two-tailed Student's *t*-test was used to determine statistical significance between means. *P* values < 0.05 are considered significant.

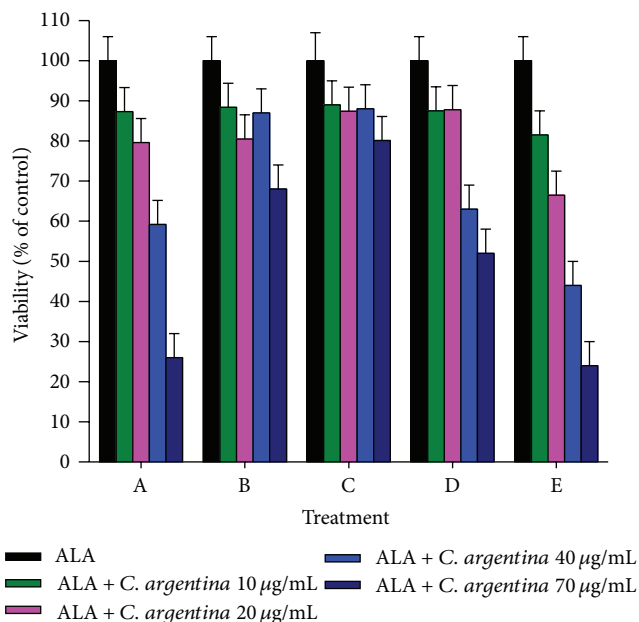


FIGURE 2: Cytotoxicity *per se* of *C. argentina* extract in LM2 cells. LM2 cells were incubated with *C. argentina* during different time periods according to each treatment described in Section 2. Cell death is referred as percentage of the nontreated control.

3. Results

In the first stage, we attempted to assess *C. argentina* dark toxicity under the different treatments (A to D), and it was known that the methanolic extract of *C. argentina* leaf is slightly toxic to LM2 cells under darkness ($CI_{50} 56 \pm 5 \mu\text{g/mL}$) [14].

Figure 2 shows cytotoxic effects of *C. argentina* under darkness (0 min irradiation). During Treatment A, at low concentrations of *C. argentina* (10 and 20 $\mu\text{g/mL}$), the toxicity *per se* was less than 20%, and at higher concentrations (40 and 70 $\mu\text{g/mL}$) *C. argentina* toxicity is proportionally increased to 41% and 75%, respectively. In Treatment B, the extract exhibits less cytotoxicity since incubation time was decreased. In Treatment C, *C. argentina* cytotoxicity is negligible since incubation time is only 10 min. Treatment D shows the highest cytotoxicity employing the highest concentrations (40 and 70 $\mu\text{g/mL}$). Treatment E involves the longest incubation time in the presence of the extract, thus showing even more marked viability decrease. To sum up, cell viability decreases as a function of the incubation time and the concentration of *C. argentina*.

In the second stage, we evaluated the effect of *C. argentina* extract to abrogate ALA-PDT induced phototoxicity in LM2 cells (Figure 3), employing ALA-PDT conditions taken from previous work [15].

C. argentina extract was added 24 h before photodynamic treatment to allow cell incorporation and during ALA-PDT (Treatment A), during ALA-PDT (Treatment B), just during the irradiation (Treatment C), after ALA-PDT (Treatment D), and before, during, and after ALA-PDT (Treatment E).

TABLE 1: Photoprotection indexes of *C. argentina* against ALA-PDT in LM2 cells after 10 min of irradiation.

<i>C. argentina</i>	10 $\mu\text{g/mL}$	20 $\mu\text{g/mL}$	40 $\mu\text{g/mL}$	70 $\mu\text{g/mL}$
Treatment A	0,02	0,10	0,92	0,84
Treatment B	0,02	0,04	0,25	0,59
Treatment C	0,02	0,02	0,17	0,21
Treatment D	0,02	0,02	0,06	0,03
Treatment E	0,02	0,06	0,69	0,74

Photoprotective indexes of *C. argentina* were determined as the ratios between % cell viability after Treatment A and % cell viability after *C. argentina* dark exposure, that is, without irradiation.

Figure 3 shows phototoxicity of ALA-PDT in the presence of *C. argentina*, normalized to the nonirradiated control. At low concentrations (10 and 20 $\mu\text{g/mL}$) of *C. argentina* under Treatment A showed no protection against ALA-PDT, but when *C. argentina* concentration was increased to 70 $\mu\text{g/mL}$, the percentage of living cells did not change upon irradiation, thus suggesting a protective effect against photosensitization.

When *C. argentina* was added only during ALA-PDT treatment (Treatment B), the photoprotection obtained was lower than that observed for Treatment A. The effect was more marked at high extract concentrations, and they increased the amount of light dose necessary to induce a certain percentage of cell death. That is, ALA-PDT with 10 min of irradiation induced 98% of cell death in absence of *C. argentina*, whereas in the presence of *C. argentina*, 40 $\mu\text{g/mL}$ and 70 $\mu\text{g/mL}$, the values decreased to 70% and 40%, respectively ($P < 0.05$).

When *C. argentina* was added just during the irradiation (Treatment C), the incubation time with the cells was not enough to allow photoprotection in all the concentration range, showing only a slight protection at the highest concentrations, achieving 83% and 79% of cell death employing 40 $\mu\text{g/mL}$ and 70 $\mu\text{g/mL}$, respectively, versus 98% of the control ($P < 0.05$).

When the extract was added after ALA-PDT and withdrawn just before MTT viability assay (Treatment D), photoprotection was not observed in all the concentration range of *C. argentina*.

In addition, when the incubation was performed before, during, and after ALA-PDT with *C. argentina* (Treatment E), the photoprotective effect was observed again, at the highest concentrations of *C. argentina*, leading only to 31% and 26% of cell death at 40 $\mu\text{g/mL}$ and 70 $\mu\text{g/mL}$, respectively, as compared to 98% of the control ($P < 0.05$).

Throughout the experiments, we noticed that the highest concentrations of *C. argentina*, the highest photoprotection indexes, independently on the treatment, employed.

We defined a photoprotection index, which allowed us to compare the protection of each treatment, independently on *C. argentina* cytotoxicity (Table 1). Indexes around 1 denote photoprotection, since they suggest no phototoxicity, whereas values around 0 indicate no protection at all. Those indexes let us know the best conditions to continue with our studies. We considered that Treatment A (incubation before and during ALA-PDT) and 40 $\mu\text{g/mL}$ of *C. argentina* would be

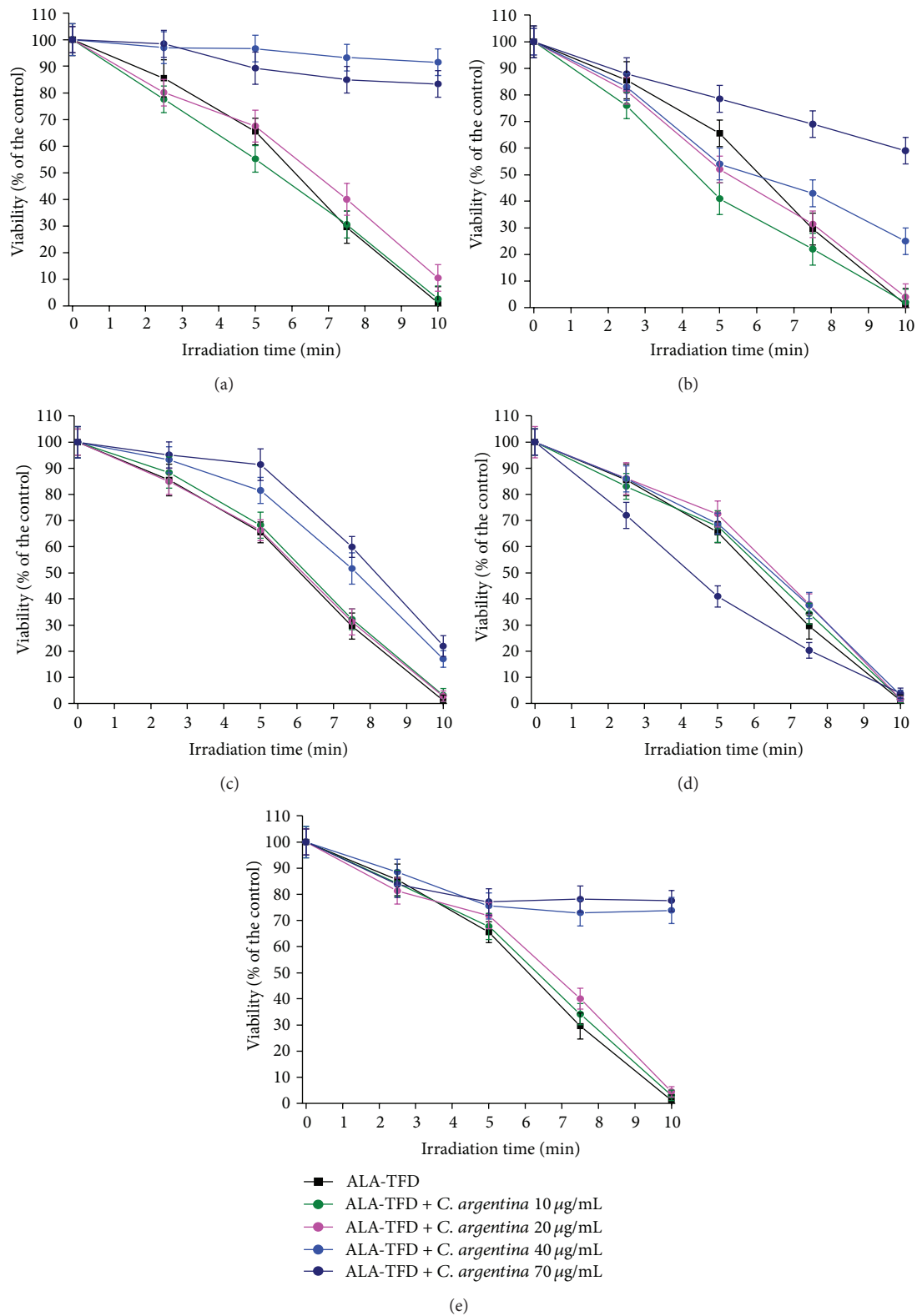


FIGURE 3: Photoprotective assays of *C. argentina* extract against ALA-PDT in LM2 cells. ALA-PDT was performed employing 0.6 mM ALA, 3 h of incubation, and different irradiation times. ((a)–(e)) correspond to the different protocols employed, as explained in Section 2. Cell viability was expressed as percentage of the control without irradiation.

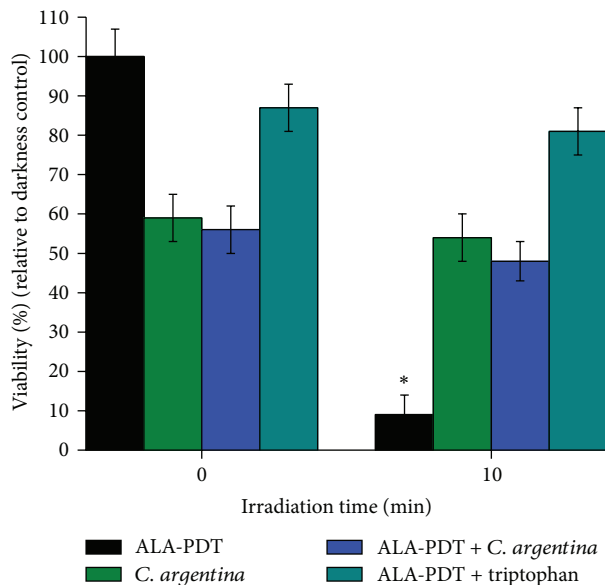


FIGURE 4: ALA-PDT of LM2 cells in the presence of *C. argentina* methanolic extract or tryptophan. Cells were subjected to ALA-PDT with 0.6 mM ALA, 3 h of incubation, and then 10 min of irradiation, in the presence of either 40 $\mu\text{g/mL}$ *C. argentina* or 8 mM tryptophan (treatment A). Viability was expressed as percentage of the control without treatment (* $P < 0,05$).

the best condition, since we obtained the higher photoprotective effect with the lower extract cytotoxicity.

Tryptophan is a well-known singlet oxygen scavenger and is a photoprotective compound against ALA-PDT [15]. We decided to compare the effect of tryptophan with the one excerpted by *C. argentina* under Treatment A. Figure 4 shows that tryptophan induces slight dark toxicity (CI_{10} for 8 mM Tryptophan), and at that concentration, it abrogated ALA-PDT effect completely. That protection was stronger than the one obtained from *C. argentina*.

With the aim to know if the photoprotective effect was exclusive to ALA-PDT or if it was independent on the PS used, we evaluated the photoprotection of *C. argentina* against PDT with the other two PSs of clinical use, Verteporfin and Temoporfin which produce singlet oxygen as the main ROS [4, 6, 7]. Figure 5 shows the effect of *C. argentina* against PDT under different schemes and extract concentrations. At low concentrations (10 and 20 $\mu\text{g/mL}$) it was not possible for the extract to excerpt photoprotection against Verteporfin or Temoporfin-PDT.

Employing 40 $\mu\text{g/mL}$ and 70 $\mu\text{g/mL}$ of the extract, a photoprotective effect against PDT with both PSs was observed. The highest photoprotection was excerpted against Verteporfin-PDT, at similar rates as compared to ALA-PDT. Cell death was reduced to 18%, which is significantly higher than 92% observed in the control of Verteporfin-PDT without extract. For Temoporfin-PDT, similar results were obtained, but achieving lower rates of photoprotection.

When we compared the photoprotective indexes of *C. argentina* against ALA, Verteporfin, or Temoporfin-PDT, we

TABLE 2: Photoprotection indexes of ALA, Verteporfin, and Temoporfin-PDT in LM2 cells.

Photosensitizer	<i>C. argentina</i> concentration			
	10 $\mu\text{g/mL}$	20 $\mu\text{g/mL}$	40 $\mu\text{g/mL}$	70 $\mu\text{g/mL}$
ALA (PpIX)	0,02	0,10	0,92	0,84
Verteporfin	0,09	0,12	0,79	0,75
Temoporfin	0,06	0,15	0,61	0,81

Photoprotective indexes of *C. argentina* were determined as the ratios between % cell viability after Treatment A and % cell viability after *C. argentina* dark exposure, that is, without irradiation.

can observe that the highest indexes were obtained against ALA-PDT (Table 2).

With the aim to test possible interferences of *C. argentina* with $^1\text{O}_2$ production during PDT, we employed Toluidine blue and Chlorin e6 as well-known PSs to induce a high singlet oxygen production (Figure 6). A dramatic decrease of $^1\text{O}_2$ production was observed in the presence of *C. argentina*, showing a direct relationship with the scavenging activity of the extract.

4. Discussion

C. argentina protected the murine LM2 adenocarcinoma cell line against PDT damage. The photoprotection was dependant on the extract concentration and the incubation time, being detectable from 40 $\mu\text{g/mL}$ onwards, and at least 3 h of cell exposure. Since *per se* cytotoxicity of the extract interferes with the analysis of the results, it was necessary to determine the best conditions to perform the studies, seeking the highest photoprotection with the lowest cytotoxicity. The best conditions were 40 $\mu\text{g/mL}$ of *C. argentina* methanolic extract and 24 h incubation before PDT and during PDT treatment.

Extract concentrations lower than 40 $\mu\text{g/mL}$ were less toxic but, however, they did not induce photoprotection. The highest concentration employed was 70 $\mu\text{g/mL}$, and even when the photoprotection was higher, *per se* cytotoxicity of *C. argentina* was extremely high (CI_{75}).

In terms of incubation time, the strongest photoprotective effects were observed at the longest incubation times, and those are 24 and 48 h. However, 48 h incubation resulted in extremely high cytotoxicity. When the extract was added just during the irradiation or after PDT treatment, no protection was observed, thus allowing us to think that the potential photoprotective compound needs longer incubation times to be taken up by the cells. This observation agrees with a previous work of our group [15] where compounds like L-tryptophan, N-acetyl-L-cystein, melatonin, L-methionine, L-cystein, mannitol, and glycine incubated before and during PDT treatment showed the best photoprotective effects. The photoprotection of *C. argentina* was comparable with that of tryptophan, which is a well-known oxygen singlet scavenger [15].

C. argentina protection against Verteporfin and Temoporfin-PDT was also observed at a lesser extent as compared to ALA-PDT. PpIX from ALA and Verteporfin are

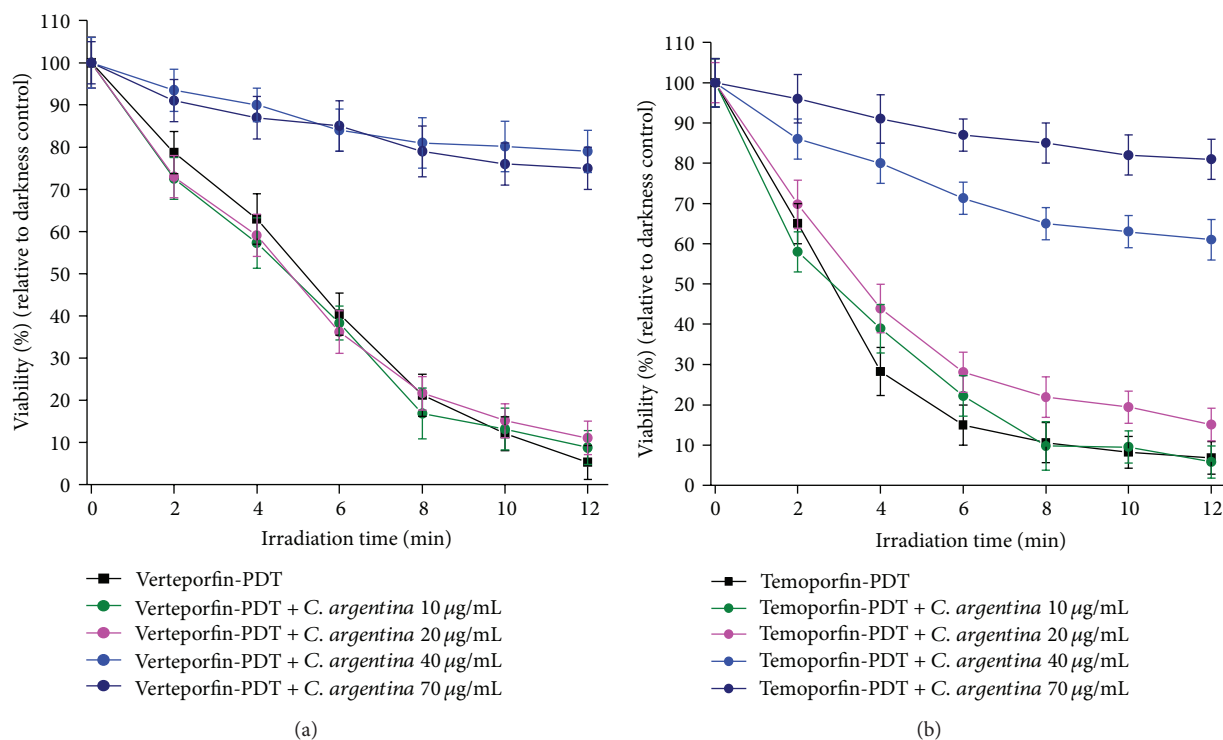


FIGURE 5: Photoprotection assays of *C. argentina* against Verteporfin and Temoporfin-PDT. LM2 cells were incubated 3 h with 3 mM Verteporfin (a) or 3 mM Temoporfin (b), and *C. argentina* was added before and during PDT (Treatment A). Viability was expressed as percentage with respect to the nonirradiated control.

accumulated mainly in mitochondria and plasma membrane. Verteporfin in addition is accumulated in the endoplasmic reticle. Temoporfin accumulates in Golgi apparatus, endoplasmic reticle, and at minor levels, in mitochondria [16–18]. The highest photoprotection against ALA and Verteporfin-mediated PDT may be related to their subcellular localization sites.

Not always the antioxidant defenses can cope with the entire PDT damage. It was reported that nasopharyngeal tumor cells that overexpress metallothioneins, which act as free radicals scavengers, were overcome by hypericin-PDT. Hypericin is a PS extract from a plant, which produces O_2^- and $^1\text{O}_2$, and upon irradiation induces necrosis in the mentioned cells [19].

Few authors reported photoprotection against PDT. Nitroimidazoles were found to be photoprotectors against PDT using Photofrin, in the emt-6 mammalian murine tumor cell line [20]. *In vivo* studies demonstrated that 1,3 diphenyl-benzofuran and tryptophan were photoprotective compounds in rats treated with PDT using hematoporphyrin as PS [21].

A disadvantage of PDT is the PS retention in sites other than tumor during long time periods after treatment, even weeks, which induces skin photosensitivity. *In vitro* and *in vivo* studies describe that singlet oxygen is responsible for cutaneous photosensitization. It is highly important to find compounds to reduce adverse effects of PDT.

Plants have biochemical systems that attenuate the harmful effects of reactive oxygen species, in particular those

generated during the photosynthesis, and xanthophylls and carotenoids account for these antioxidant defenses involved. Other compounds are able to synthesize molecules which respond to biotic and abiotic stress, commonly called secondary metabolites. Some of these compounds have been reported as photoprotectors and/or antioxidants. Beta-carotene and flavonoids usually are in chloroplast membranes to protect the damage caused by $^1\text{O}_2$, in a particular way to protect against lipid photoperoxidation. Scavenger effects of lipid plant extracts against $^1\text{O}_2$ were detected [22].

Polyphenols synthesized in higher plants, in response to stress, constitute also part of their antioxidant defense system [23]. Curcumin extracted from *Curcuma longa* is able to inhibit the apoptosis generated by oxidant damage of $^1\text{O}_2$ during PDT treatment in A431 epidermoid carcinoma cells [24].

UV and psoralen therapy is a kind of photochemotherapy which uses UV radiation instead of visible light as it is used in PDT. The use of plant extracts for UV protection is widely known. The extract of *Polypodium leucotomos* administrated orally or topically, resulted in photoprotection in Langerhans cells against UV and psoralens employed in vitiligo treatment [25].

Employing qualitative tests to identify organic molecule functional groups, we detected the presence of anthraquinones, saponins, carbohydrates, and tanins in *C. argentina* extract. However, we do not know the exact composition of *C. argentina* and its relationship with its photoprotective properties.

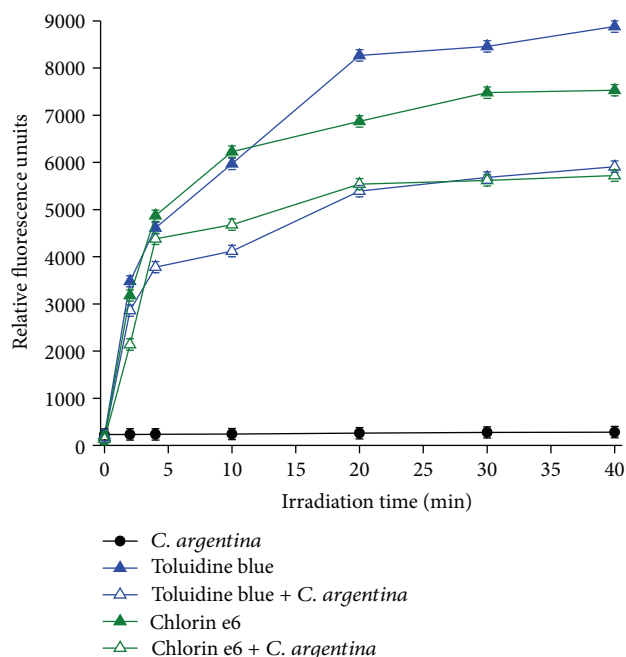


FIGURE 6: *C. argentina* as scavenger of singlet oxygen produced by Toluidine blue and Chlorin e6-PDT. Singlet oxygen production was quantified using a fluorescent specific probe at different times of PDT employing Toluidine blue and Chlorin e6 as photosensitizers.

To sum up, our results indicate that *C. argentina* extract protects mammalian tumor cells against PDT effects, and it interferes with singlet oxygen production during photodynamic treatment. We propose this will be a promising agent to protect skin from adverse photosensitivity. It is our hope to continue this work with *in vivo* studies and purification of the extract with the aim to isolate the compound responsible for the photoprotection and further studies in normal skin tissue are needed to ascertain that the plant *C. argentina* may be employed in the prevention from side effects caused by PDT. Our findings would be useful in the design of ointments to protect normal skin against the undesirable effects of PDT.

Abbreviations

ALA: 5-Aminolevulinic acid
 MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
 PDT: Photodynamic therapy
 PpIX: Protoporphyrin IX
 PS: Photosensitizer
 PSs: Photosensitizers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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